Cell Host & Microbe
Resource

An Infection-Relevant Transcriptomic Compendium for *Salmonella enterica* Serovar Typhimurium

Carsten Kröger,[1] Aoife Colgan,[1] Shabarinath Srikumar,[1] Kristian Händler,[1] Sathesh K. Sivasankaran,[1] Disa L. Hammarlöf,[4] Rocío Canals,[4] Joe E. Grissom,[2] Tyrrell Conway,[2] Karsten Hokamp,[3] and Jay C.D. Hinton[1,4,*]
[1]Department of Microbiology, School of Genetics & Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland
[2]Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA
[3]Department of Genetics, School of Genetics & Microbiology, Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2, Ireland
[4]Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK
*Correspondence: jay.hinton@liverpool.ac.uk
http://dx.doi.org/10.1016/j.chom.2013.11.010

SUMMARY

Bacterial transcriptional networks consist of hundreds of transcription factors and thousands of promoters. However, the true complexity of transcription in a bacterial pathogen and the effect of the environments encountered during infection remain to be established. We present a simplified approach for global promoter identification in bacteria using RNA-seq-based transcriptomic analyses of 22 distinct infection-relevant environmental conditions. Individual RNA samples were combined to identify most of the 3,838 *Salmonella enterica* serovar Typhimurium promoters in just two RNA-seq runs. Individual in vitro conditions stimulated characteristic transcriptional signatures, and the suite of 22 conditions induced transcription of 86% of all *S.* Typhimurium genes. We highlight the environmental conditions that induce the *Salmonella* pathogenicity islands and present a small RNA expression landscape of 280 sRNAs. This publicly available compendium of environmentally controlled expression of every transcriptional feature of *S.* Typhimurium constitutes a useful resource for the bacterial research community.

INTRODUCTION

Thousands of bacterial genome sequences are now available, generating exciting insights into bacterial speciation and the evolution of organisms like *Salmonella* (Okoro et al., 2012). However, we do not know the function of a high proportion of bacterial genes, partly because we lack knowledge of when and how the genes are expressed. Transcription produces RNA molecules that either have a direct function or are translated into a protein. As scientists quest to understand how bacterial pathogens cause disease, complete catalogs of RNA species must be assembled.

The 2,400 serovars of *Salmonella enterica* subspecies I possess a range of abilities to cause disease in mammalian and avian hosts. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a key serovar that has serious impact upon human health around the world and carries clusters of virulence genes that permit colonization of the gastrointestinal tract, invasion of epithelial cells, and replication within macrophages. An intricate transcriptional control system is required to ensure that virulence genes are transcribed in the right place at the right time (Fàbrega and Vila, 2013). This choreography of transcription allows the bacteria to be pathogenic while remaining fit enough to compete effectively with the native microbiota, resulting in a remarkably complex transcriptome.

Our understanding of bacterial transcription has advanced in great leaps in the past decade. It is more than fifty years since the discovery of the first operon showed that bacterial genes could be transcribed as polycistronic mRNAs (Jacob and Monod, 1961). The advent of deep sequencing (Margulies et al., 2005) and the development of RNA-seq (Nagalakshmi et al., 2008) now allow transcription to be studied at the level of the individual nucleotide in the context of the millions of nucleotides that comprise the genome. It is now possible to identify the primary transcriptome, which is a catalog of all expressed RNA molecules that carry a 5′-triphosphate group (Sharma et al., 2010). However, until now microbial transcriptomes have been studied under relatively few environmental conditions (Cho et al., 2009; Kröger et al., 2012; Mandlik et al., 2011; Sharma et al., 2010; Toledo-Arana et al., 2009). Because bacterial gene expression is remarkably sensitive to environmental conditions such as temperature, oxygen, and stressors, a complete picture of the transcriptome requires the combination of information from bacteria exposed to different environmental factors. Hence, we devised a strategy for RNA sequencing of pooled RNA samples that can be obtained from any number of growth conditions and can be readily reproduced in laboratories across the world.

To demonstrate our approach, we used a suite of 22 infection-relevant conditions designed to simulate the environmental signals encountered by *S.* Typhimurium in its host. This comprehensive view includes 3,838 promoters and the 280 small RNA species (sRNAs) that make up the RNA world of this bacterium and reveals the true complexity of the bacterial transcriptome. We present a simplified method for the identification of most promoters and sRNAs, in just two RNA-seq experiments, that is applicable to diverse bacterial systems.

Table 1. The Suite of 22 Infection-Relevant Growth Conditions

Condition name	Growth description
EEP	Growth in Lennox broth to OD_{600} 0.1.
MEP	Growth in Lennox broth to OD_{600} 0.3.
LEP	Growth in Lennox broth to OD_{600} 1.0.
ESP	Growth in Lennox broth to OD_{600} 2.0.
LSP	Growth in Lennox broth to OD_{600} 2.0, followed by a further 6 hr growth.
25°C	Growth in Lennox broth to OD_{600} 0.3 at 25°C.
Cold shock (15°C)	Growth in Lennox broth to OD_{600} 0.3, then transfer from 37°C to 15°C for 10 min.
Temp10	Growth in Lennox broth to OD_{600} 0.3 at 25°C, then transfer to 37°C for 10 min.
Temp20	Growth in Lennox broth to OD_{600} 0.3 at 25°C, then transfer to 37°C for 20 min.
pH3 shock	Growth in Lennox broth to OD_{600} 0.3, then cells were harvested by centrifugation, resuspended in fresh Lennox (pH 3.0), and grown for an additional 10 min.
pH5.8 shock	Growth in Lennox broth to OD_{600} 0.3, then cells were harvested by centrifugation, resuspended in fresh Lennox (pH 5.8), and grown for additional 10 min.
NaCl shock	Growth in Lennox broth to OD_{600} 0.3, then addition of NaCl to a final concentration of 0.3 M for 10 min.
Bile shock	Growth in Lennox broth to OD_{600} 0.3, then addition of bile to a final concentration of 3% for 10 min.
LowFe^{2+} shock	Growth in Lennox broth to OD_{600} 0.3, then addition of 2,2′-dipyridyl to a final concentration of 0.2 mM for 10 min.
Anaerobic shock	Growth in Lennox broth to OD_{600} 0.3 (50 ml), then filled into 50 ml closed Falcon tube and incubated without agitation at 37°C for 30 min.
Anaerobic growth	Static growth in Lennox broth to OD_{600} 0.3 in a completely filled and closed 50 ml Falcon tube.
Oxygen shock	Static growth in Lennox broth to OD_{600} 0.3 in a completely filled and closed 50 ml Falcon tube, then 15 min aerobic growth (baffled flask, 250 rpm).
NonSPI2	Growth in PCN medium (pH 7.4, 25 mM P_i) to OD_{600} 0.3.
InSPI2	Growth in PCN medium (pH 5.8, 0.4 mM P_i) to OD_{600} 0.3.
InSPI2 LowMg^{2+}	Growth in PCN (InSPI2) medium with 10 μM $MgSO_4$ to OD_{600} 0.3.
Peroxide shock (InSPI2)	Growth in PCN (InSPI2) to OD_{600} 0.3, then addition of H_2O_2 to final concentration of 1 mM H_2O_2 for 12 min.
Nitric oxide shock (InSPI2)	Growth in PCN medium (InSPI2) to OD_{600} 0.3, then addition of 250 μM Spermine NONOate for 20 min.
Pool	RNA from all 22 conditions pooled.

S. Typhimurium is exposed to a variety of environmental stressors during the infection of the mammalian host (Hébrard et al., 2011). These 22 conditions reflect aspects of the infection process and are based on media published by several laboratories over the past decade (Bourret et al., 2008; Löber et al., 2006; McHugh et al., 2003; Prouty and Gunn, 2000; Wright et al., 2009). Precise details, such as the waterbath used to agitate cultures, are in Experimental Procedures and in Data Set S1.

The transcriptomic data can be viewed in the context of the chromosome with an interactive online browser (http://tinyurl.com/HintonLabSalComJbrowse), and we have created a user-friendly online tool that allows the broader microbial community to interrogate the expression profiles of *S.* Typhimurium genes with ease (http://tinyurl.com/HintonLabSalCom). These data reveal the highly environmentally sensitive gene expression programs that underpin the pathogenesis of *S.* Typhimurium.

RESULTS AND DISCUSSION

Growth Conditions and the RNA Pool Rationale

S. Typhimurium can adapt its transcriptional program within 4 min when it encounters a new environment (Rolfe et al., 2012), and this ability is thought to contribute to the success of the bacterium as a pathogen. Transcriptional control is mediated by a large group of regulatory proteins and small regulatory RNAs that have been identified in *S.* Typhimurium over recent decades (Fàbrega and Vila, 2013; Hébrard et al., 2012; McClelland et al., 2001). It has become clear that *Salmonella* gene expression is exquisitely sensitive to the environmental perturbations that the bacteria experience during the process of infecting a mammalian host (Hébrard et al., 2011). We designed a suite of infection-relevant in vitro growth situations that represents aspects of the different environments encountered by the bacterium during pathogenesis. Ten of these situations involve short environmental shocks induced by transfer into particular media for 10–20 min and represent the immediate gene expression response to particular stressors. The conditions that are relevant to colonization of the mammalian gastrointestinal tract include exposure to acid (pH 3.0), anoxia, bile, increased osmolarity, Fe^{2+} limitation, three different temperatures, and five stages of bacterial growth in rich medium. The conditions that reflect the intracellular life of *S.* Typhimurium inside mammalian macrophages are exposure to peroxide or nitric oxide and combinations of moderate acid (pH 5.8), phosphate limitation, or Mg^{2+} limitation. The suite of 22 growth conditions and relevant abbreviations is detailed in Data Set S1 and in Table 1. To generate a complete

picture of the *Salmonella* transcriptome under these conditions, RNA from each of the 22 conditions was analyzed individually and also combined into a single sample (RNA pool).

These experiments were carried out using *S*. Typhimurium 4/74, a strain that promises to become the ideal model for the ST19 sequence type of *S*. Typhimurium. The ST19 clade is responsible for significant levels of gastroenteritis in developed countries. Strain 4/74 (Jones et al., 1988), also known as S2337 or S2337/65, was originally isolated from a calf with salmonellosis (Rankin and Taylor, 1966) and is highly virulent in cattle, pigs, chickens, and mice (Chaudhuri et al., 2013). *S*. Typhimurium 4/74 was used by Bruce Stocker as the parent of the well-characterized SL1344 strain. SL1344 is an auxotrophic strain that was constructed by introducing the *hisG46* mutation into 4/74 via a two-step transduction with phage P22 (Hoiseth and Stocker, 1981). The 4/74 strain differs from SL1344 by only eight SNPs (four of which lie in the 8 kb region around the *hisG* gene), and the complete genome sequence of both strains is available (Kröger et al., 2012; Richardson et al., 2011). Note that strain 4/74 is a prototroph that possesses a functional histidine biosynthetic pathway that is important for infection of certain mammalian cells (Henry et al., 2005).

Large-Scale RNA-Seq Analysis of *S*. Typhimurium and dRNA-Seq of Individual and Pooled RNA Samples

Total RNA was isolated from *S*. Typhimurium strain 4/74 cultured in 22 environmental conditions and used to construct cDNA libraries for RNA-seq (Kröger et al., 2012). To identify promoters in *S*. Typhimurium, we used the well-established differential RNA-seq method (dRNA-seq). The dRNA-seq technique involves the use of terminator exonuclease (TEX) to selectively digest 5′-monophosphorylated RNA species and to enrich for primary transcripts that possess a 5′-triphosphate prior to cDNA library construction (Sharma et al., 2010). Subsequent RNA-seq analysis allows reliable identification of transcriptional start sites (TSS) at single-nucleotide resolution, by comparison with a standard, non-TEX-treated RNA-seq library. We previously used this method to identify 1,873 TSS in a single condition (early stationary phase; ESP), for the closely related *S*. Typhimurium strain SL1344 (Kröger et al., 2012).

A total of ~602 million sequence reads (487 million reads for 22 RNA-seq libraries and 121 million reads for dRNA-seq libraries; sequencing statistics are detailed in Data Set S1) were generated on the Illumina HiSeq platform, with an average of > 16 million reads per library. The pooled RNA sample was sequenced to a depth of 20 million reads (RNA-seq) and 19 million reads (dRNA-seq). Approximately 94% of sequenced reads were mapped onto the *S*. Typhimurium 4/74 genome (Data Set S1). The RNA samples were not ribo-depleted prior to cDNA library construction, and we discarded the majority of sequence reads that corresponded to rRNA and tRNA genes by limiting our analysis to the ~214 million sequence reads that mapped to a single location on the chromosome (Data Set S1). A recent study showed that sequencing of 5–10 million non-rRNA reads provides coverage of the vast majority of bacterial transcripts (Haas et al., 2012). Because our data set contained an average of 6.4 million uniquely mapped reads per sample, it is a robust representation of the bacterial transcriptome in each of the 22 conditions. The proportion of mRNAs, small RNAs (sRNAs), antisense RNAs, and structural/housekeeping RNAs is shown in Figure S1A, and the visualization of the normalized mapped sequence reads can be examined at http://tinyurl.com/HintonLabSalComJbrowse.

The Majority of *S*. Typhimurium Genes Are Expressed in at Least One Environment

To relate mapped sequence reads to gene expression, the absolute and relative levels of gene expression were determined from the RNA-seq data by the transcripts per million (TPM) approach (Wagner et al., 2012, 2013) (Data Set S2). The expression data have been compiled into SalCom, a compendium of *Salmonella* transcriptomic data that can be interrogated at http://tinyurl.com/HintonLabSalCom. We determined the number of *S*. Typhimurium genes that were expressed in at least one sample condition (Experimental Procedures). An average of 63% of genes was expressed in each individual condition, while ~74% of all genes were expressed in the RNA pool (Figure S1B). This fits with microarray-based transcriptomic data from *E. coli* that detected transcripts for 60% of the 4,290 *E. coli* coding sequences in a single environment (Arfin et al., 2000). It was striking that a much lower proportion of genes was expressed at late stationary phase (24%), reflecting the reduced level of transcription (Proshkin et al., 2010).

The majority of *Salmonella* genes (86%) were expressed in at least one of the 22 environmental conditions. Each environment generates a distinct transcriptional signature that reflects the mechanisms used by *Salmonella* to adapt to stress. Examples include the upregulation of the detoxification and repair genes that respond to exposure of *Salmonella* to peroxide (*katG*, *ahpC*, and *oxyS*) (Wright et al., 2009) or to nitric oxide (*hmpA*, *norV*, and *ytfE*) (Bourret et al., 2008).

To investigate transcriptomic changes in the core genome of *S.* Typhimurium, we determined the number of genes that were up- and downregulated for the majority of environmental conditions (>3-fold, Figures 1A and 1B). The biggest changes in gene expression occurred upon the osmotic, anaerobic, nitric oxide, and peroxide shocks, when the expression of ~15%–25% of *Salmonella* chromosomal genes changed within about 10 min. To investigate whether these brief environmental shocks induced an individual response to specific stressors or activated a more general stress response, we compared the genes that were more than 3-fold upregulated during the LowFe^{2+}, bile, anaerobic, and NaCl shocks with mid-exponential phase (MEP) and compared the peroxide and nitric oxide shocks to InSPI2 (an acidic phosphate-limiting minimal media that induces *Salmonella* pathogenicity island (SPI) 2 transcription (Löber et al., 2006) (Figures 1C and 1D). We found that individual environmental shocks caused the upregulation of specific sets of genes. However, the Venn diagrams did not identify a common response to multiple stressors. We deduce that the response of *Salmonella* to these six environmental shocks involves specialized transcriptional programs that control the processes required for the bacteria to survive in each condition.

We identified 672 genes that were not expressed in the suite of conditions (TPM value < 10; Wagner et al., 2013). Most of these genes encode proteins involved in shaping cell surface structure, cell motility, and protein secretion (in particular the type VI secretion system [T6SS]) and catabolic pathways for

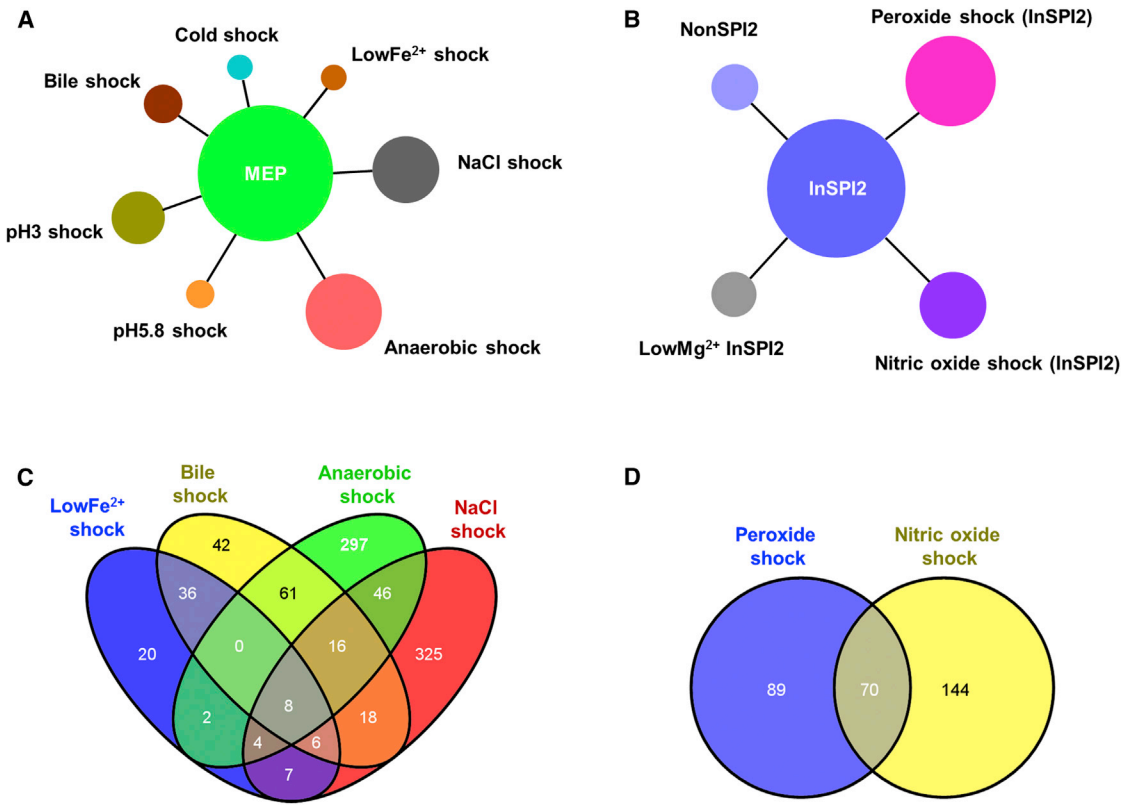

Figure 1. Visualization of the Extent of Gene Expression Changes and Comparison of the Upregulated Genes that Respond to Brief Environmental Shocks

(A–D) The size of the area of the outer circles corresponds to the number of genes which are > 3-fold up- and downregulated in comparison to MEP (A) (cold shock, 109 genes; LowFe^{2+}, 109 genes; NaCl shock, 737 genes; anaerobic shock, 951 genes; bile shock, 245 genes; pH3 shock, 465 genes; pH 5.8 shock, 134 genes) and InSPI2 (B) (NonSPI2, 354 genes; LowMg^{2+}, 328 genes; nitric oxide shock, 737 genes; peroxide shock, 1,334 genes). The size of the area of each central comparator circle corresponds to the number of genes that are expressed (TPM > 10), and these are MEP (3,008 genes) and InSPI2 (3,134 genes). The Venn diagrams show the number of genes that are > 3-fold upregulated in the environmental shocks in comparison to MEP (C) and InSPI2 (D). The Venn diagrams were created using Venny (http://bioinfogp.cnb.csic.es/tools/venny/). See also Data Set S2.

the utilization of alternative carbon sources (i.e., arabinose, N-acetyl glucosamine, rhamnose, fucose, or *myo*-inositol (Figure S1C). The identity of most of the nonexpressed genes was expected because the suite of environments involved planktonic bacteria and was based on either rich L-broth or a single defined minimal medium with glucose as the sole carbon source. Identifying the 672 nonexpressed genes will allow us to devise additional environmental conditions that will promote transcription of most of these genes.

Promoters Identified from the RNA Pool Represent the 22 Environmental Conditions

To identify TSS, five dRNA-seq libraries were constructed from RNA isolated from *S.* Typhimurium strain 4/74 grown in four distinct environmental conditions, namely MEP, ESP, late stationary phase (LSP), and InSPI2, plus the RNA pool. The precise locations of the mapped sequence read counts were normalized and visualized using the Integrated Genome Browser (IGB) (Nicol et al., 2009; Experimental Procedures). TSS were only assigned when sequence reads were enriched in the dRNA-seq libraries compared with the RNA-seq library for the same environmental condition and corresponded to an expressed transcript, or if they had been experimentally confirmed before by others (criteria detailed in Experimental Procedures; Data Set S3). This conservative approach ruled out transcripts originating from spurious promoter-like sequences that are only rarely conserved in enteric bacteria (Raghavan et al., 2012).

By focusing on five environmental conditions, we identified 3,838 TSS, which were used to identify distinct promoter categories (Figure 2A) (Kröger et al., 2012; Sharma et al., 2010). Although automated TSS assignment can now alleviate the laborious process of manual TSS identification (Dugar et al., 2013), we found that manual curation remains mandatory for the generation of a high-quality list of TSS. Two TSS (a secondary TSS of *hilD* and the primary TSS of *stpA*) were confirmed by 5′RACE (Figure S3). We also mapped the exact TSS of the promoter of the SPI2-encoded *ssaR* gene, which has been reported to be SsrB dependent (Figure S2; Tomljenovic-Berube et al., 2010). More than 95% of the previously identified 1,873 TSS (from ESP; Kröger et al., 2012) exactly match the TSS reported here.

We used these robust TSS data to determine whether the RNA pool could be used to represent the entire *S.* Typhimurium primary transcriptome. Comparison with individual dRNA-seq experiments from MEP, ESP, LSP, and InSPI2 revealed that

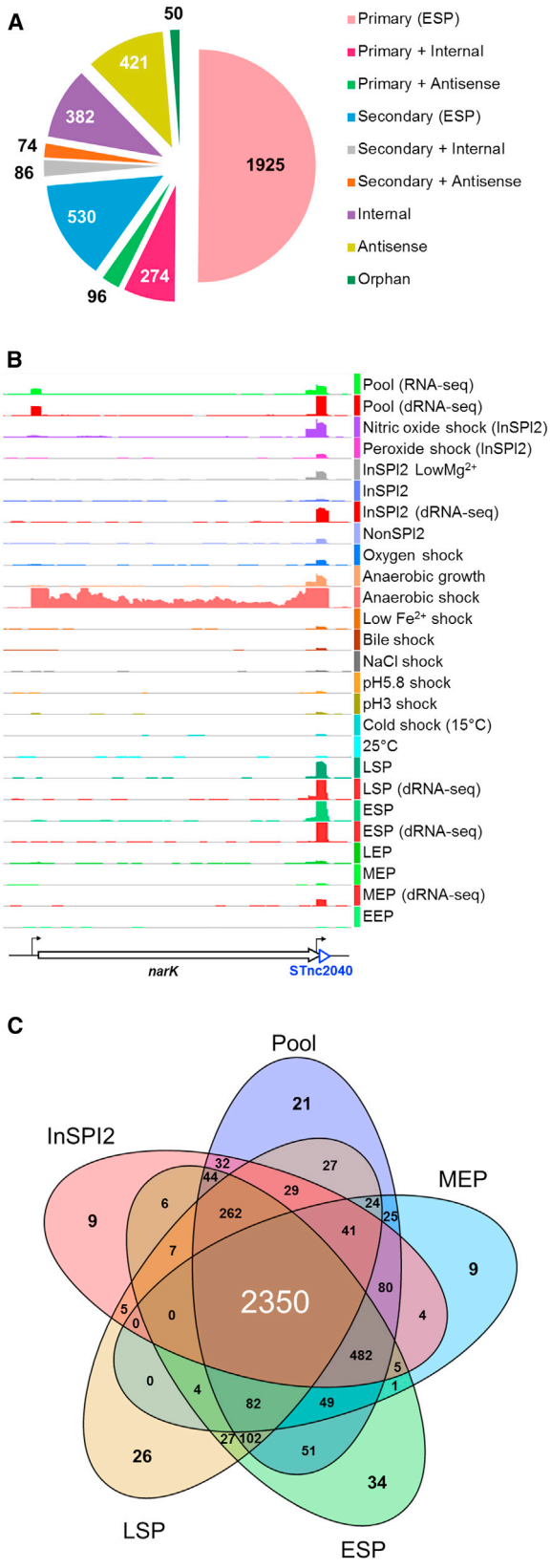

Figure 2. Classification of Transcriptional Start Sites of *S.* Typhimurium

(A) The identified TSS were grouped into nine promoter categories according to Sharma et al. (2010).

(B) Mapped sequence reads for the *narK* and *STnc2040* genes presented in the IGB browser. The scale is 0–100 normalized reads for every sample. The figure shows the TSS of *narK*, which is only expressed in anaerobic shock and is present in the pool.

(C) TSS distribution from five dRNA-seq experiments (MEP, ESP, LSP, InSPI2, and RNA pool). See also Data Set S3.

3,701 TSS (96% of all identified TSS) could be identified by comparison of the dRNA-seq and RNA-seq analysis of the RNA pool. The sensitivity of the RNA pool was remarkable, as many TSS in the RNA pool sample were only expressed in one of the 22 conditions. This is exemplified by the *narK* gene, which is only expressed during anaerobic shock (Figure 2B). A core of 2,350 TSS was transcribed in all five conditions (Figure 2C), and 279 TSS were present in the pool, but not in ESP. The small proportion of TSS that were identified in individual dRNA-seq experiments but not detected in the RNA pool did not pass our stringent assignment criteria because of low expression (TPM $\leq$ 10). We predict that most of these TSS would be assigned if the depth of sequencing of the RNA pool was increased.

In summary, the pooling of RNA from a diverse range of environmental conditions allows the promoter regions to be identified for the vast majority of bacterial genes in just two RNA-seq libraries. This approach could be readily applicable to other bacteria and should be extended by isolating RNA from bacteria grown in any creative way to maximize gene expression.

Transcriptional Organization and Expression Analysis of *Salmonella* Pathogenicity Islands

The *Salmonella* genus carries a number of genomic islands that are integral to *Salmonella* virulence (Sabbagh et al., 2010). The online tool (http://tinyurl.com/HintonLabSalCom) permits "wild card" searches to visualize the expression profiles of hundreds of genes in a single heat map, using terms such as *prg** or *sii**. We used this functionality to analyze the expression profiles of *Salmonella* SPIs and identified characteristic expression signatures in the suite of environmental conditions.

Previous reviews of the regulation of SPI1 genes (Golubeva et al., 2012) and SPI2 genes (Fass and Groisman, 2009) have described crosstalk between pathogenicity islands, such as the substantial coexpression of SPI4 genes with SPI1 (Gerlach et al., 2007; Main-Hester et al., 2008). However, most published findings were made by focusing on the expression of selected genes under few growth conditions, and it was not clear how the transcription of the individual SPIs relate to each other. We addressed this by interrogating our RNA-seq-based global gene expression data from infection-relevant conditions, which reveals precise details about transcripts at the nucleotide level. Here, we present the transcriptional landscape of SPI1 (Figure 3), SPI2 (Figure S2A), and other SPI islands (SPI3, SPI4, SPI5, SPI11, SPI12, SPI16; Figures S2–S4). Heat maps of expression of all *S.* Typhimurium pathogenicity islands are presented in Figure 4 and in Figures S5 and S6.

It is clear that SPI1 and SPI2 show distinct expression profiles in the suite of environmental conditions. These patterns likely

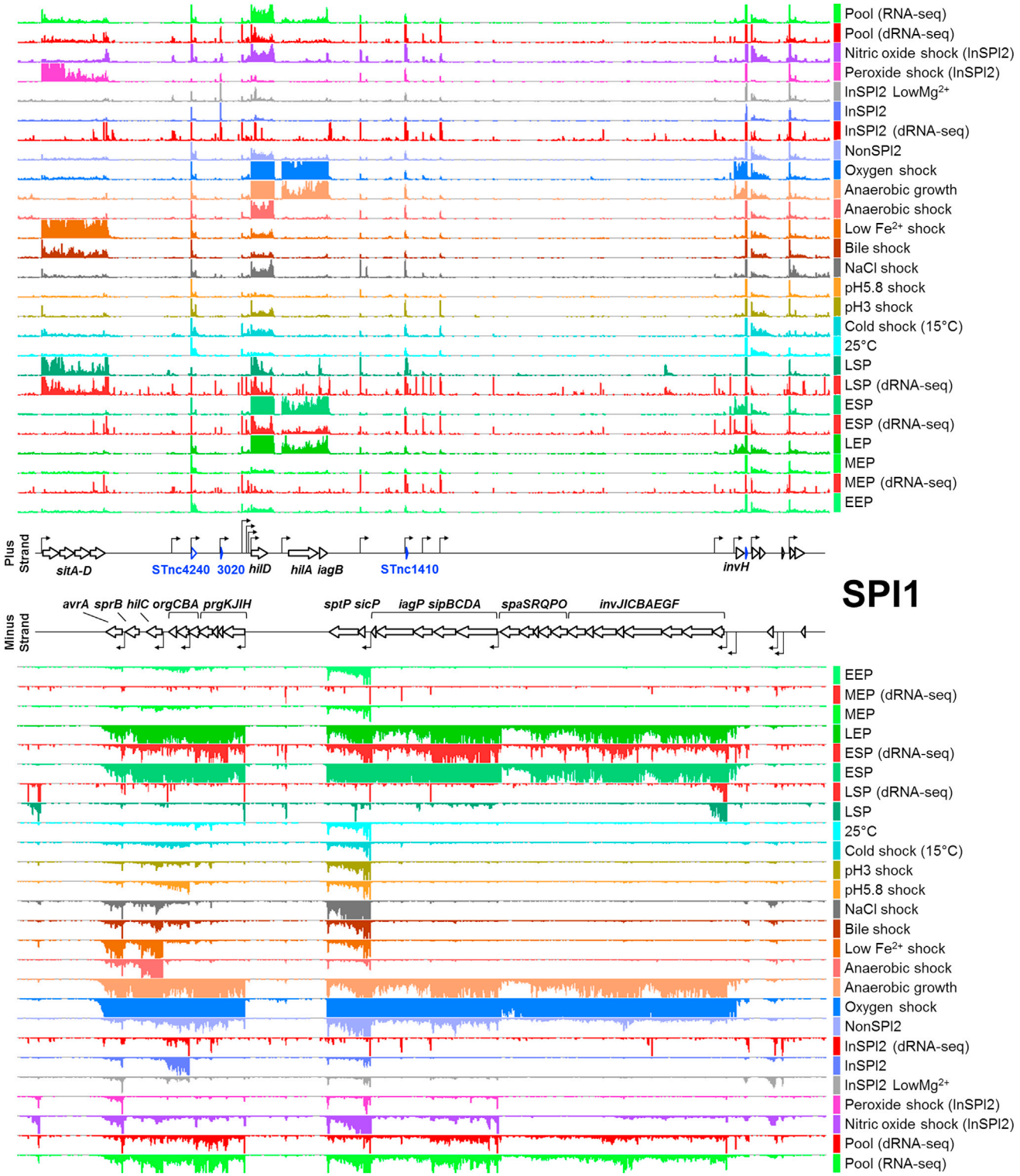

Figure 3. Visualization of Mapped Sequence Reads in the SPI1 Pathogenicity Island

Protein coding gene names are labeled in black, small RNA gene names in blue. TSS are marked by arrows. All dRNA-seq libraries are shown in red. The figure was prepared with the IGB browser, and the scale is 0–100 normalized reads for every sample. See also Figure S2.

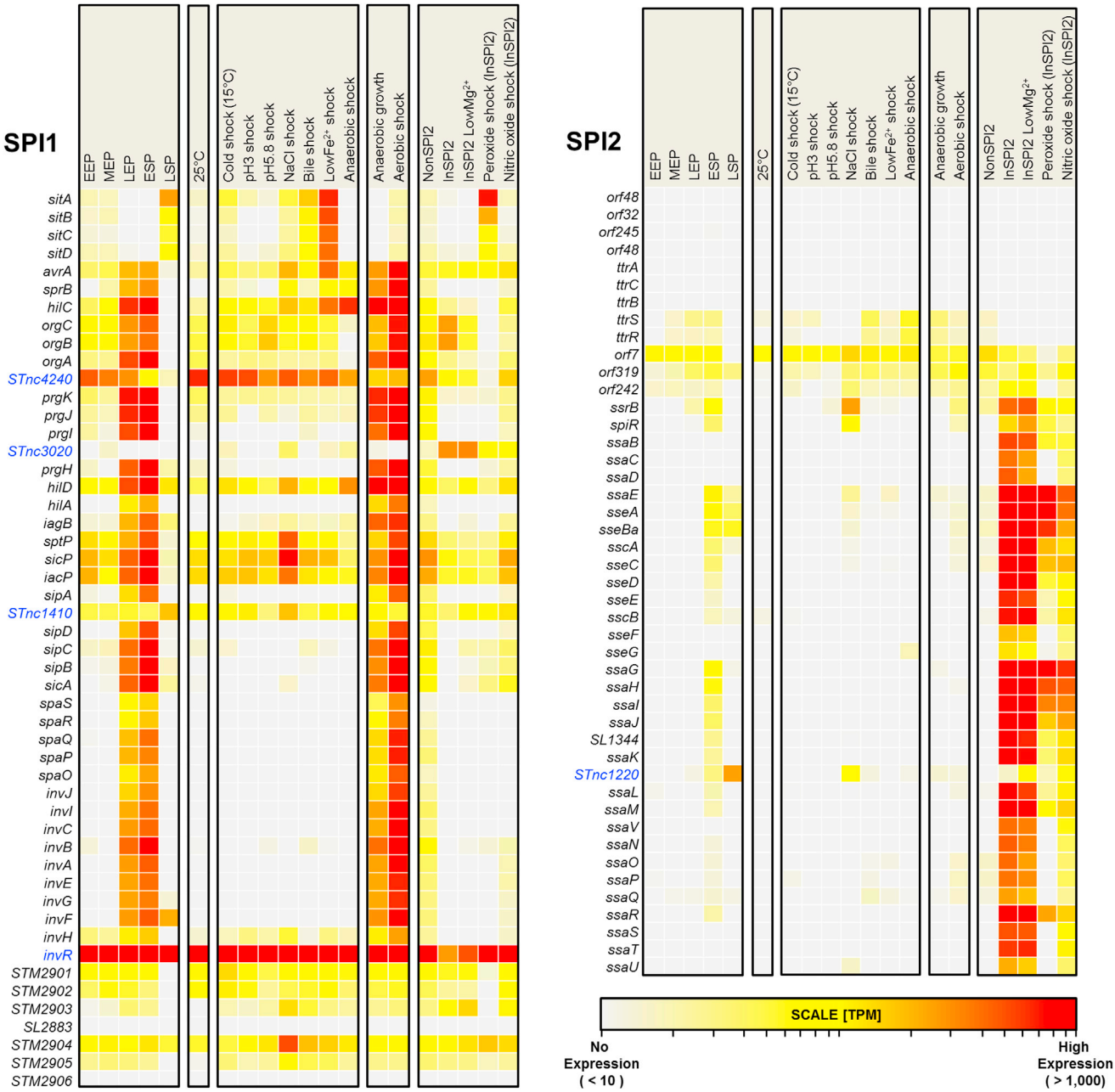

Figure 4. Absolute Gene Expression for SPI1 and SPI2

The heat map shows the absolute gene expression of SPI1 (left) and SPI2 (right) genes in TPM values. Coding gene names are labeled in black, small RNA gene names in blue. The TPM values are in Data Set S2. See also Figure S5.

reflect the predominantly extracellular (SPI1) or intracellular (SPI2) function of each pathogenicity island and represent signature profiles that can be used to discover other coding genes that show a SPI1- or SPI2-like pattern of expression (see below). The compendium of data presented here offer a comprehensive resource that describes the transcriptional organization and expression profiles of *Salmonella* genes. As well as defining the promoters and transcriptional architecture of the *S.* Typhimurium chromosome, we anticipate that the ability to visualize expression profiles of individual *S.* Typhimurium coding genes and sRNAs will be of value to scientists interested in *Salmonella* virulence and gene regulation.

Identifying SPI1-like and SPI2-like Transcriptional Signatures and Effector Expression Profiles

Expression profiling of the *S.* Typhimurium transcriptome showed distinct patterns for SPI1 and SPI2 in the ESP, in anaerobic growth and oxygen shock conditions, and in the InSPI2 media. To define the transcriptional signature of the two pathogenicity islands, we used individual genes to reflect the

expression of SPI1 (*prgH*; encodes the inner membrane needle complex of SPI1) and SPI2 (*ssaG*; encodes part of the type 3 secretion complex of SPI2). These archetypical genes are required for the function of either SPI1 or SPI2 and report the output of the regulatory hierarchy that controls each type III secretion system (T3SS). Correlative analyses of the entire transcriptome identified 41 genes with a SPI1-like pattern of expression (Pearson correlation coefficient > 0.8, Data Set S2). Twenty seven of those genes are located within SPI1, while 14 genes are located elsewhere on the chromosome and mostly code for SPI1 effector proteins (see below) or are part of SPI4. Expression of two genes that are not T3SS-associated and do not encode effector proteins (STM4313 and *lpxR* [STM1328]) correlated with other SPI1 genes. The uncharacterized protein STM4313 has been reported to be expressed in a SPI1-like pattern (Wang et al., 2004), while the LPS-modifying enzyme LpxR has not. Transposon-mediated mutation of both these genes was recently shown to impair the fitness of *Salmonella* to colonize the gut of calves, pigs, and chickens, which is typically a characteristic of SPI1 mutations (Chaudhuri et al., 2013).

Sixty six genes show a SPI2-like pattern of expression (Pearson correlation coefficient > 0.8, Data Set S2) and include 27 of the SPI2 island genes and 39 coding genes located elsewhere on the chromosome. Of these 39 genes, 16 genes are likely to be regulated by the SPI2-encoded regulator SsrB, which binds their promoter regions (Tomljenovic-Berube et al., 2010) (Data Set S2). SPI5 carries genes that respond in either a SPI1- or SPI2-like expression pattern (Figures S3A, S5A, and S6A). We confirm that *sopB* and *pipC* are highly expressed in a SPI1-like fashion (LEP, ESP, anaerobic growth, and oxygen shock), while the *pipB* gene shows a SPI2-like expression profile (Knodler et al., 2002). We speculate that some of the uncharacterized genes that show SPI1- or SPI2-like expression patterns will encode virulence factors.

Salmonella effector proteins are synthesized within the bacterium and translocated into the cytosol of mammalian cells. Effectors are classified into three categories, which are either secreted via the SPI1 T3SS, the SPI2 T3SS, or both translocases. The biological relevance of SPI1- and SPI2-like transcriptional signatures becomes apparent when considering expression of these effector proteins. Figure 5 shows that genes that encode effectors translocated via SPI1 have a SPI1-like expression profile (average Pearson correlation coefficient 0.95 ± 0.03 [*prgH*]). Similarly, genes encoding effectors translocated via SPI2 showed a SPI2-like expression profile (average Pearson correlation coefficient 0.8 ± 0.2 [*ssaG*]). Intriguingly, the eight effector genes that encode proteins secreted by both SPI1 and SPI2 T3SS showed a range of distinct expression profiles that were neither SPI1- or SPI2-like (average Pearson correlation coefficient 0.30 ± 0.43 [*prgH*] and 0.26 ± 0.31 [*ssaG*]). Our findings show that, as has been seen in other bacteria (Thattai, 2013), a correlative approach will simplify the assignment of *Salmonella* gene function.

Expression Landscape of *Salmonella* sRNAs

Small regulatory RNAs are an emerging class of posttranscriptional regulators, and *Salmonella* has become a model organism for the characterization of sRNA function in pathogenic Gram-negative bacteria (Hébrard et al., 2012; Papenfort and Vogel, 2010). The criteria for the designation of small RNA candidates are described in the Supplemental Experimental Procedures section. By investigating the transcriptome of *Salmonella* in the suite of conditions, we identified 78 small RNA candidates, increasing the number of sRNAs found in *Salmonella* Typhimurium to a total of 280 (Data Set S4).

The expression profiles of the 280 sRNAs were studied in the suite of conditions. We present the expression of six well-characterized sRNAs (Figure 6A; DapZ, OxyS, RybB, RprA, RyhB-1, and InvR). The pie charts show the reported patterns of upregulation of DapZ in ESP (Chao et al., 2012), OxyS in peroxide shock (Altuvia et al., 1997), RybB in LSP (Papenfort et al., 2006), RprA in NaCl shock (Majdalani et al., 2001), RyhB-1 in LowFe^{2+} shock (Padalon-Brauch et al., 2008), and InvR in ESP (Pfeiffer et al., 2007). The analysis also reveals the high level of expression of RprA at LSP and RybB in LowMg^{2+} and the discovery that InvR and DapZ are expressed during anaerobic growth and during subsequent oxygen shock. We confirmed the condition-dependent upregulation of 12 small RNAs by northern blotting. For example, STnc3120 was upregulated by NaCl shock and STnc3080 responded to Fe^{2+} availability (Figure 6B and Figure S7). We present a comprehensive "sRNA expression landscape" of all 280 sRNAs that can be accessed via a dedicated link on the SalCom website (http://tinyurl.com/HintonLabSalCom).

Global Classification of *Salmonella* sRNAs

A canonical model of posttranscriptional regulation by sRNAs involves translational repression by a sRNA binding to its target mRNA, which typically is facilitated by the RNA chaperone Hfq. However, an increasing number of sRNA mechanisms and functions are now being reported (Lalaouna et al., 2013). To aid future mechanistic analyses, we categorized the 280 sRNAs into five classes (intergenic, antisense, 5′- or 3′-end-located, intragenic) according to their chromosomal location in relation to nearby coding genes (Figure 6C). Small RNAs were regarded as antisense if they overlap more than 50% of a coding gene located on the opposite strand (note that "antisense" refers only to the location and not to the mechanism of regulation). We find that the majority of sRNAs (60%) are transcribed from intergenic regions. The second largest group of sRNAs is located at the 3′ end of a coding gene (20%), which represents a genomic reservoir of regulatory sRNAs (Chao et al., 2012), while 14% are transcribed from the antisense strand. Only 5% are located at the 5′ end of a coding region, but this number might not be completely representative. Although RNA-seq is the ideal way to find sRNAs, sRNAs associated with the 5′ UTR and regulatory elements (i.e., riboswitches) are particularly hard to identify because the mapped sequence reads of the sRNA and the 5′ UTR of a coding region appear to overlap.

To determine which of the 280 sRNAs are likely to have a regulatory function, we drew upon the extensive literature for *E. coli* and *S.* Typhimurium that shows that regulatory sRNAs generally require Hfq as a chaperone to mediate interactions with target mRNAs (Vogel and Luisi, 2011). Fortunately, two coimmunoprecipitation-based studies have identified the cellular RNA bound to Hfq in *S.* Typhimurium SL1344 (Chao et al., 2012; Sittka et al., 2008). Raw sequence data from Chao et al. (GEO database accession number GSE38884) were analyzed to find which of the total of 280 sRNAs strongly

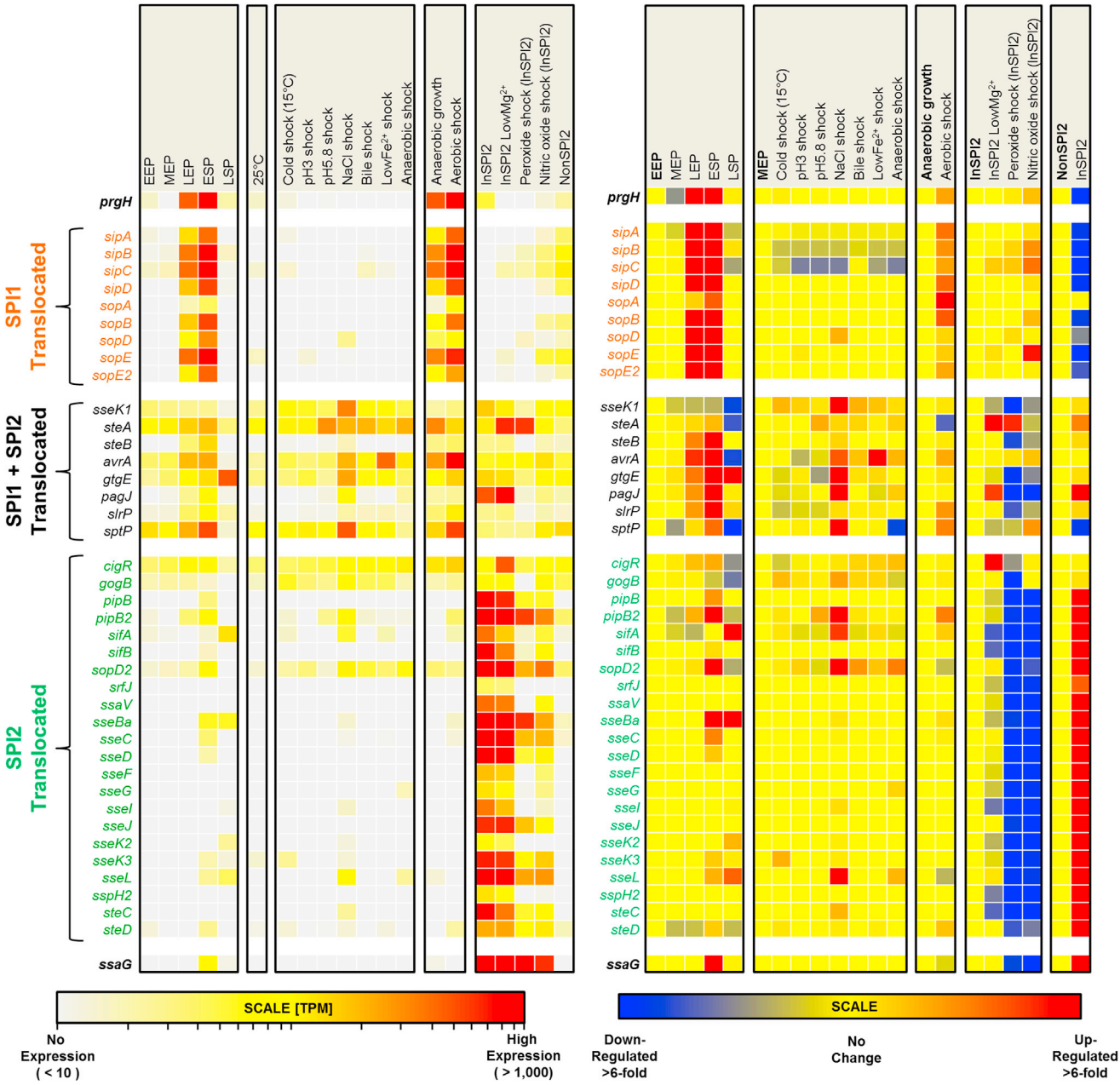

Figure 5. Absolute and Relative Expression of Genes Encoding Effector Proteins

Genes that encode proteins translocated by the SPI1 or SPI2 T3SS, or both, are labeled in orange (SPI1), green (SPI2), and black (both), respectively (Buckner et al., 2011; Figueira et al., 2013; Heffron et al., 2011). See also Data Set S2.

bound Hfq. The results are shown in the context of the chromosomal location of each sRNA (Figure 6D). A total of 115 (41%) sRNAs bound strongly to Hfq in at least one condition using the data from published Hfq-coIP-seq data sets (Hfq-enrichment factor > 5; Data Set S4) (Chao et al., 2012; Sittka et al., 2008). These include sRNAs proven to regulate target genes via an Hfq-mediated mechanism in *Salmonella*, including SgrS, RybB, and SdsR (Fröhlich et al., 2012; Papenfort et al., 2006, 2012, 2013). In Data Set S4, we list the 115 Hfq-associated sRNAs that are bound by Hfq and are likely to control the sRNA-mediated transcriptional network of *Salmonella* by a *trans*-acting mechanism.

Based on the enrichment of primary transcripts in the dRNA-seq experiments, we found that most sRNAs (71%) are transcribed as primary transcripts (Figure 6E). The 56 sRNAs located in 3′ regions of coding genes are thought to either arise from a processing event of the mRNA of the upstream ORF or to be transcribed from a separate promoter. Intriguingly, the latter type can be regulated independently from the 5′-located coding region, exemplified by the sRNA DapZ (Chao et al., 2012).

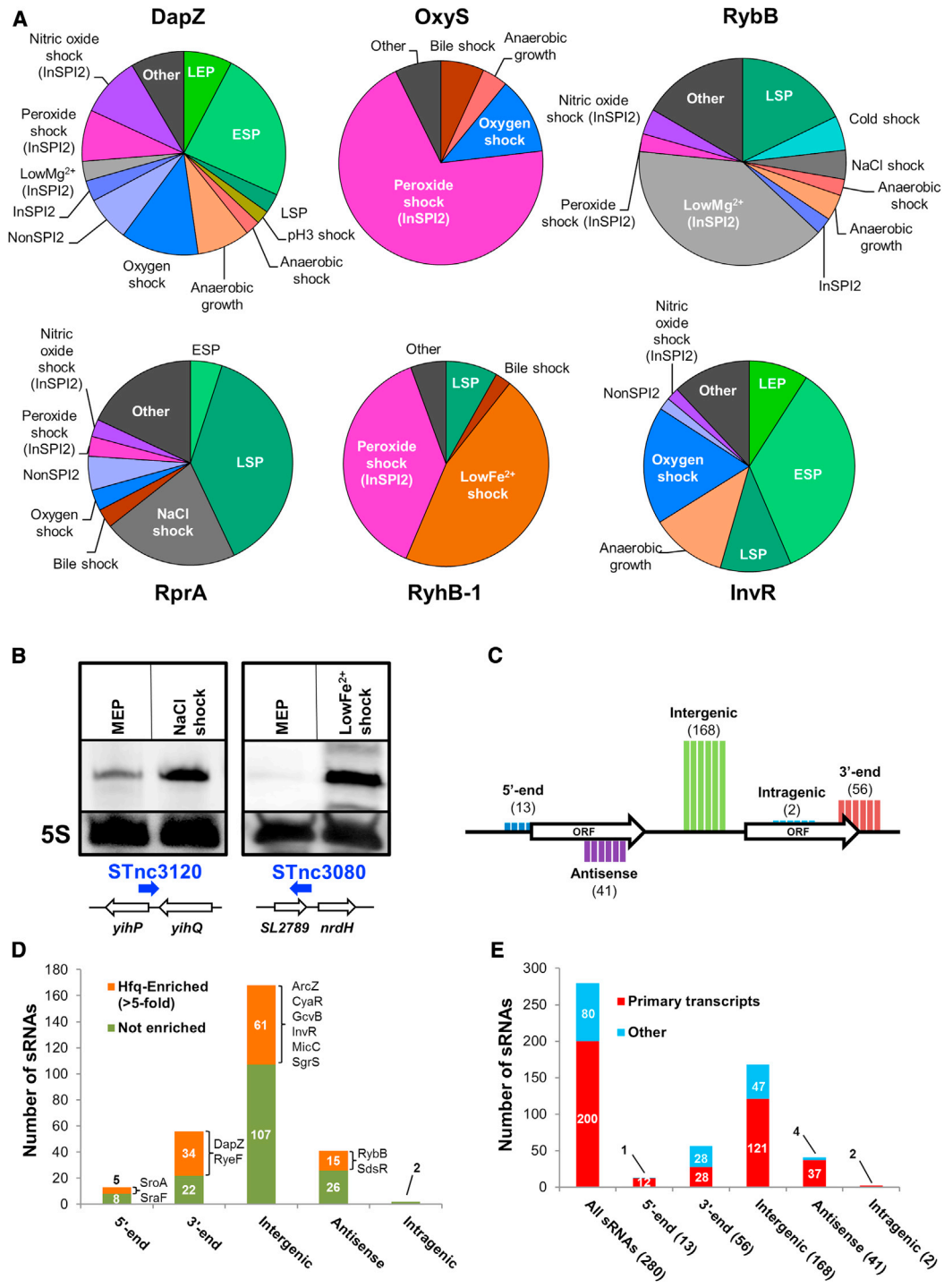

Figure 6. Expression Analyses and Classification of sRNAs of *S.* Typhimurium

(A) Expression of six small RNAs: DapZ, OxyS, RybB, RprA, RyhB-1, and InvR. The pie charts show the proportion of sRNA gene expression across different conditions (based on TPM values).

(B) Northern blots of two sRNAs (STnc3120, STnc3080). STnc3120 is upregulated in response to NaCl shock, while STnc3080 is upregulated upon $LowFe^{2+}$ shock. 5S rRNA served as a loading control.

(C) Genomic location of sRNAs relative to coding genes. The height of each bar reflects the number of sRNAs in each category (Data Set S4).

(D) Hfq binding of sRNAs in the context of their genomic location. Hfq binding was assessed using the Hfq-coIP data produced by Chao et al. (2012) and Sittka et al. (2008) (Data Set S4). A selection of sRNAs reported to bind Hfq is shown in the figure.

(E) Assessment of sRNA transcripts. Based on enrichment in the dRNA-seq experiments, the bar chart shows the number of sRNAs that are unequivocally primary transcripts in the context of their genomic location (red proportion of each bar). See also Figure S7.

Overall, we found that half of the 3′-located sRNAs possess their own promoters (Figure 6E).

Concluding Remarks

Transcription of DNA into RNA is a fundamental process for all living organisms. In bacteria, the transcriptional output is tightly regulated and is modulated by environmental perturbations. RNA-seq has become the best tool to study bacterial transcriptomes because of its high resolution, reliability, and dynamic range. We present a simplified approach to identify most bacterial promoters and sRNAs, by pooling RNA from many different growth conditions to create just two cDNA libraries (dRNA-seq and RNA-seq).

During infection of a host, *Salmonella* encounters many different and changing environments to which the bacteria must adapt. To study the transcriptional response of *Salmonella* to its environment, we devised a suite of 22 infection-relevant conditions. These multicondition data allow the identification of characteristic transcriptional signatures for each environment and the use of the correlation of gene expression as a powerful tool to infer gene function. The suite of environmental conditions described here should accelerate the functional characterization of *Salmonella* genes and are currently being used by us for transcriptional network analyses.

The data show that 86% of all *Salmonella* genes are expressed in at least one environment, which is comparable to the level of expression of yeast genes (90%) (Wilhelm et al., 2008) and the human genome (75%) (Djebali et al., 2012). Clearly, the majority of genes are transcribed in all biological systems, and the expression of *Salmonella* genes are highly responsive to environmental perturbations.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and RNA Isolation

Salmonella enterica serovar Typhimurium strain 4/74 was used throughout the study; is highly virulent in chickens, calves, and pigs (Chaudhuri et al., 2013); and is the parental strain of SL1344 (Hoiseth and Stocker, 1981). Cells were grown overnight in 5 ml Lennox (L-) broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and diluted the next morning (1:1,000) in 250 ml flasks in 25 ml L-broth and grown at 37°C and 220 rpm. The suite of 22 environmental conditions is described in Table 1 and in more detail in Data Set S1 and in the Supplemental Experimental Procedures. All agitated bacterial cultures were grown in 250 ml flasks containing 25 ml media in a New Brunswick Innova 3400 water bath prior to RNA isolation. Transcription and RNA turnover were stopped using 2/5 volumes of the cell suspension using ice-cold "stop solution" (19% ethanol, 1% acidic phenol [pH 4.3]) and incubated on ice for 30 min to prevent RNA degradation (Tedin and Bläsi, 1996). RNA was isolated using TRIzol (Invitrogen) and DNase I digestion was conducted as described earlier (Kröger et al., 2012).

Preparation of cDNA Libraries and Illumina Deep Sequencing

For RNA-seq, cDNA libraries were generated from DNase-digested total RNA samples without ribosomal RNA depletion or enrichment, and sequencing was performed by Vertis Biotechnologie AG (Freising, Germany), as described earlier (Kröger et al., 2012). For dRNA-seq, RNA samples were digested with Terminator Exonuclease (TEX) prior to cDNA library preparation. The cDNA libraries were sequenced on Illumina HiSeq 2000 system by Vertis Biotechnologie AG (Freising, Germany).

Mapping of Sequenced Reads and Differential Gene Expression

Sequence reads were mapped against the *S.* Typhimurium 4/74 reference genome using Segemehl with accuracy set to 100% (Hoffmann et al., 2009; Richardson et al., 2011). To increase mapping quality, unmapped reads were sequentially truncated by one nucleotide from the 3′ end of the read and then mapped again. This process was iterated until a read mapped into a single location on the chromosome, or a minimal read length of 20 nucleotides was reached. Expression values for coding genes and noncoding sRNAs were calculated using the Transcript Per Million (TPM) method (Data Set S2) (Wagner et al., 2012, 2013).

We confirmed the reproducibility of the RNA-seq-based transcriptomic data set by analyzing biological replicates for ESP, LSP, and InSPI2 conditions and calculating the following correlation coefficients of log2-transformed TPM (>10)-based expression: ESP = 0.986, LSP = 0.973, InSPI2 = 0.991. Technical replicates were produced from MEP (three technical replicates) and ESP (two technical replicates), and TPM values were calculated from the combined replicates. Correlation coefficients of log2-transformed TPM (>10)-based expression of the technical replicates were also calculated: MEP $\geq$ 0.99 for all combinations; ESP = 0.996.

To distinguish whether a gene is expressed or not, a cut-off was defined based on indicator genes that have been previously shown not to be expressed in a particular condition, i.e., genes needed for utilization of alternative carbon sources. For instance, *iol* genes needed for *myo*-inositol utilization are only expressed when *myo*-inositol is present in the growth medium (Kröger and Fuchs, 2009). The average gene expression of eight *iol* genes that are essential for *myo*-inositol degradation is TPM = 3.7 ± 3.0. Based on this observation, we set a conservative cut-off for genes that are not expressed as a TPM value of 10. Genes with a TPM value > 10 were regarded as expressed. As reads for duplicated coding genes (paralogs) or duplicated small RNAs cannot be mapped unequivocally, these genes appear in our analysis with no mapped reads. For visualization of sequence reads in IGB and JBrowse, the read depth was adjusted in relation to the cDNA library with the lowest number of reads (Skinner et al., 2009). This was achieved by dividing the read coverage at each genomic position by the library size and multiplying it by the size of the smallest library (cold shock [15°C]). Numbers of sequence reads are shown in Data Set S1.

Identification of SPI1- and SPI2-like Transcriptional Signatures

The use of similarity metrics to identify classes of coexpressed genes from a transcriptomic compendium is well established (Hughes et al., 2000). The Pearson correlation was used to identify genes that showed a similar expression profile over the 22 environmental conditions using GeneSpring GX7.3 software. The SPI1- and SPI2-like transcriptional signatures identified genes that shared a correlation coefficient of > 0.8 with either *prgH* (SPI1) or *ssaG* (SPI2).

Identification of Transcriptional Start Sites

The rationale for TSS assignation was that each previously unidentified TSS must be both enriched in at least one of the dRNA-seq libraries and linked to an expressed transcript in the corresponding RNA-seq experiment. Only transcripts matching both requirements were assigned as a TSS. In addition, we included TSS that have been described earlier (Kröger et al., 2012). To determine whether a TSS was present in particular conditions or not, a set of TPM values was calculated for the first ten nucleotides of each transcript. A TSS was only regarded as expressed when the TPM value was > 10. Enrichment of TSS in the dRNA-seq data in relation to the corresponding RNA-seq data was determined visually for each individual TSS in IGB using tracks of normalized sequence reads, as described above.

ACCESSION NUMBERS

The RNA-seq data can be downloaded as raw reads (.fastq file format) and normalized "IGB files" (.gr file format) ready to load into the IGB browser from the GEO database accession number GSE49829.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, Supplemental Experimental Procedures, and four Excel Data Sets and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.11.010.

AUTHOR CONTRIBUTIONS

C.K. and J.C.D.H. designed the study. C.K. and A.C. performed wet-lab experiments. S.K.S. and K. Hokamp performed mapping of RNA-seq data and TPM calculations. C.K., K. Hokamp, and J.C.D.H. analyzed and interpreted the data. A.C., S.S., K. Händler, S.K.S., D.L.H., R.C., and T.C. contributed to data analysis and interpretation. K. Hokamp created the websites. J.E.G. and T.C. contributed code and design for the websites. A.C., D.L.H., R.C., T.C., and K. Hokamp revised the manuscript. C.K. and J.C.D.H. wrote the manuscript.

ACKNOWLEDGMENTS

We are grateful to Jörg Vogel, Kai Papenfort, Konrad Förstner, Cynthia M. Sharma, and Yanjie Chao for sharing unpublished data and ideas; to Mark Stevens and Tim Wallis for discussions about strain 4/74; and to Jonathan Hardy, Ken Sanderson, and Susan Hoiseth for details of the construction of SL1344 in Bruce Stocker's lab. We appreciate Katie Breen and Jane Twohig for technical support. The authors thank José L. Puente, Andrew D.S. Cameron, and Ian R. Monk for their insight and Stephen Fitzgerald for sharing primers. We also thank Fritz Thümmler (Vertis Biotechnologie AG) for excellent cDNA construction. The work was financially supported by Science Foundation Ireland (Grant 08/IN.1/B2104) and the University of Liverpool.

Received: October 2, 2013
Revised: November 13, 2013
Accepted: November 21, 2013
Published: December 11, 2013

REFERENCES

Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L., and Storz, G. (1997). A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell *90*, 43–53.

Arfin, S.M., Long, A.D., Ito, E.T., Tolleri, L., Riehle, M.M., Paegle, E.S., and Hatfield, G.W. (2000). Global gene expression profiling in *Escherichia coli* K12. The effects of integration host factor. J. Biol. Chem. *275*, 29672–29684.

Bourret, T.J., Porwollik, S., McClelland, M., Zhao, R., Greco, T., Ischiropoulos, H., and Vázquez-Torres, A. (2008). Nitric oxide antagonizes the acid tolerance response that protects *Salmonella* against innate gastric defenses. PLoS ONE *3*, e1833.

Buckner, M.M., Croxen, M.A., Arena, E.T., and Finlay, B.B. (2011). A comprehensive study of the contribution of *Salmonella enterica* serovar Typhimurium SPI2 effectors to bacterial colonization, survival, and replication in typhoid fever, macrophage, and epithelial cell infection models. Virulence *2*, 208–216.

Chao, Y., Papenfort, K., Reinhardt, R., Sharma, C.M., and Vogel, J. (2012). An atlas of Hfq-bound transcripts reveals 3′ UTRs as a genomic reservoir of regulatory small RNAs. EMBO J. *31*, 4005–4019.

Chaudhuri, R.R., Morgan, E., Peters, S.E., Pleasance, S.J., Hudson, D.L., Davies, H.M., Wang, J., van Diemen, P.M., Buckley, A.M., Bowen, A.J., et al. (2013). Comprehensive assignment of roles for *Salmonella typhimurium* genes in intestinal colonization of food-producing animals. PLoS Genet. *9*, e1003456.

Cho, B.K., Zengler, K., Qiu, Y., Park, Y.S., Knight, E.M., Barrett, C.L., Gao, Y., and Palsson, B.O. (2009). The transcription unit architecture of the *Escherichia coli* genome. Nat. Biotechnol. *27*, 1043–1049.

Djebali, S., Davis, C.A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A., Lagarde, J., Lin, W., Schlesinger, F., et al. (2012). Landscape of transcription in human cells. Nature *489*, 101–108.

Dugar, G., Herbig, A., Förstner, K.U., Heidrich, N., Reinhardt, R., Nieselt, K., and Sharma, C.M. (2013). High-resolution transcriptome maps reveal strain-specific regulatory features of multiple *Campylobacter jejuni* isolates. PLoS Genet. *9*, e1003495.

Fàbrega, A., and Vila, J. (2013). *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin. Microbiol. Rev. *26*, 308–341.

Fass, E., and Groisman, E.A. (2009). Control of *Salmonella* pathogenicity island-2 gene expression. Curr. Opin. Microbiol. *12*, 199–204.

Figueira, R., Watson, K.G., Holden, D.W., and Helaine, S. (2013). Identification of *Salmonella* pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of *S. enterica* serovar typhimurium: implications for rational vaccine design. MBio *4*, e00065.

Fröhlich, K.S., Papenfort, K., Berger, A.A., and Vogel, J. (2012). A conserved RpoS-dependent small RNA controls the synthesis of major porin OmpD. Nucleic Acids Res. *40*, 3623–3640.

Gerlach, R.G., Jäckel, D., Geymeier, N., and Hensel, M. (2007). *Salmonella* pathogenicity island 4-mediated adhesion is coregulated with invasion genes in *Salmonella enterica*. Infect. Immun. *75*, 4697–4709.

Golubeva, Y.A., Sadik, A.Y., Ellermeier, J.R., and Slauch, J.M. (2012). Integrating global regulatory input into the *Salmonella* pathogenicity island 1 type III secretion system. Genetics *190*, 79–90.

Haas, B.J., Chin, M., Nusbaum, C., Birren, B.W., and Livny, J. (2012). How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes? BMC Genomics *13*, 734.

Hébrard, M., Kröger, C., Sivasankaran, S.K., Händler, K., and Hinton, J.C. (2011). The challenge of relating gene expression to the virulence of *Salmonella enterica* serovar Typhimurium. Curr. Opin. Biotechnol. *22*, 200–210.

Hébrard, M., Kröger, C., Srikumar, S., Colgan, A., Händler, K., and Hinton, J.C. (2012). sRNAs and the virulence of *Salmonella enterica* serovar Typhimurium. RNA Biol. *9*, 437–445.

Heffron, F., Niemann, G., Yoon, H., Kidwai, A., Brown, R.N.E., McDermott, J.D., Smith, R., and Adkins, J.N. (2011). *Salmonella*-secreted Virulence Factors. In Salmonella: From Genome to Function, S. Porwollik, ed. (Norfolk, UK: Caister Academic Press).

Henry, T., García-Del Portillo, F., and Gorvel, J.P. (2005). Identification of *Salmonella* functions critical for bacterial cell division within eukaryotic cells. Mol. Microbiol. *56*, 252–267.

Hoffmann, S., Otto, C., Kurtz, S., Sharma, C.M., Khaitovich, P., Vogel, J., Stadler, P.F., and Hackermüller, J. (2009). Fast mapping of short sequences with mismatches, insertions and deletions using index structures. PLoS Comput. Biol. *5*, e1000502.

Hoiseth, S.K., and Stocker, B.A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature *291*, 238–239.

Hughes, T.R., Marton, M.J., Jones, A.R., Roberts, C.J., Stoughton, R., Armour, C.D., Bennett, H.A., Coffey, E., Dai, H., He, Y.D., et al. (2000). Functional discovery via a compendium of expression profiles. Cell *102*, 109–126.

Jacob, F., and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. *3*, 318–356.

Jones, P.W., Collins, P., and Aitken, M.M. (1988). Passive protection of calves against experimental infection with *Salmonella typhimurium*. Vet. Rec. *123*, 536–541.

Knodler, L.A., Celli, J., Hardt, W.D., Vallance, B.A., Yip, C., and Finlay, B.B. (2002). *Salmonella* effectors within a single pathogenicity island are differentially expressed and translocated by separate type III secretion systems. Mol. Microbiol. *43*, 1089–1103.

Kröger, C., and Fuchs, T.M. (2009). Characterization of the *myo*-inositol utilization island of *Salmonella enterica* serovar Typhimurium. J. Bacteriol. *191*, 545–554.

Kröger, C., Dillon, S.C., Cameron, A.D., Papenfort, K., Sivasankaran, S.K., Hokamp, K., Chao, Y., Sittka, A., Hébrard, M., Händler, K., et al. (2012). The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. Proc. Natl. Acad. Sci. USA *109*, E1277–E1286.

Lalaouna, D., Simoneau-Roy, M., Lafontaine, D., and Massé, E. (2013). Regulatory RNAs and target mRNA decay in prokaryotes. Biochim. Biophys. Acta *1829*, 742–747.

Löber, S., Jäckel, D., Kaiser, N., and Hensel, M. (2006). Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. Int. J. Med. Microbiol. *296*, 435–447.

Main-Hester, K.L., Colpitts, K.M., Thomas, G.A., Fang, F.C., and Libby, S.J. (2008). Coordinate regulation of *Salmonella* pathogenicity island 1 (SPI1) and SPI4 in *Salmonella enterica* serovar Typhimurium. Infect. Immun. *76*, 1024–1035.

Majdalani, N., Chen, S., Murrow, J., St John, K., and Gottesman, S. (2001). Regulation of RpoS by a novel small RNA: the characterization of RprA. Mol. Microbiol. *39*, 1382–1394.

Mandlik, A., Livny, J., Robins, W.P., Ritchie, J.M., Mekalanos, J.J., and Waldor, M.K. (2011). RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. Cell Host Microbe *10*, 165–174.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature *437*, 376–380.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., et al. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature *413*, 852–856.

McHugh, J.P., Rodríguez-Quinoñes, F., Abdul-Tehrani, H., Svistunenko, D.A., Poole, R.K., Cooper, C.E., and Andrews, S.C. (2003). Global iron-dependent gene regulation in *Escherichia coli*. A new mechanism for iron homeostasis. J. Biol. Chem. *278*, 29478–29486.

Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M., and Snyder, M. (2008). The transcriptional landscape of the yeast genome defined by RNA sequencing. Science *320*, 1344–1349.

Nicol, J.W., Helt, G.A., Blanchard, S.G., Jr., Raja, A., and Loraine, A.E. (2009). The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics *25*, 2730–2731.

Okoro, C.K., Kingsley, R.A., Connor, T.R., Harris, S.R., Parry, C.M., Al-Mashhadani, M.N., Kariuki, S., Msefula, C.L., Gordon, M.A., de Pinna, E., et al. (2012). Intracontinental spread of human invasive *Salmonella* Typhimurium pathovariants in sub-Saharan Africa. Nat. Genet. *44*, 1215–1221.

Padalon-Brauch, G., Hershberg, R., Elgrably-Weiss, M., Baruch, K., Rosenshine, I., Margalit, H., and Altuvia, S. (2008). Small RNAs encoded within genetic islands of *Salmonella typhimurium* show host-induced expression and role in virulence. Nucleic Acids Res. *36*, 1913–1927.

Papenfort, K., and Vogel, J. (2010). Regulatory RNA in bacterial pathogens. Cell Host Microbe *8*, 116–127.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., and Vogel, J. (2006). SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. Mol. Microbiol. *62*, 1674–1688.

Papenfort, K., Podkaminski, D., Hinton, J.C., and Vogel, J. (2012). The ancestral SgrS RNA discriminates horizontally acquired *Salmonella* mRNAs through a single G-U wobble pair. Proc. Natl. Acad. Sci. USA *109*, E757–E764.

Papenfort, K., Sun, Y., Miyakoshi, M., Vanderpool, C.K., and Vogel, J. (2013). Small RNA-Mediated Activation of Sugar Phosphatase mRNA Regulates Glucose Homeostasis. Cell *153*, 426–437.

Pfeiffer, V., Sittka, A., Tomer, R., Tedin, K., Brinkmann, V., and Vogel, J. (2007). A small non-coding RNA of the invasion gene island (SPI-1) represses outer membrane protein synthesis from the *Salmonella* core genome. Mol. Microbiol. *66*, 1174–1191.

Proshkin, S., Rahmouni, A.R., Mironov, A., and Nudler, E. (2010). Cooperation between translating ribosomes and RNA polymerase in transcription elongation. Science *328*, 504–508.

Prouty, A.M., and Gunn, J.S. (2000). *Salmonella enterica* serovar typhimurium invasion is repressed in the presence of bile. Infect. Immun. *68*, 6763–6769.

Raghavan, R., Sloan, D.B., and Ochman, H. (2012). Antisense transcription is pervasive but rarely conserved in enteric bacteria. MBio *3*, e00156–e00112.

Rankin, J.D., and Taylor, R.J. (1966). The estimation of doses of *Salmonella typhimurium* suitable for the experimental production of disease in calves. Vet. Rec. *78*, 706–707.

Richardson, E.J., Limaye, B., Inamdar, H., Datta, A., Manjari, K.S., Pullinger, G.D., Thomson, N.R., Joshi, R.R., Watson, M., and Stevens, M.P. (2011). Genome sequences of *Salmonella enterica* serovar typhimurium, Choleraesuis, Dublin, and Gallinarum strains of well- defined virulence in food-producing animals. J. Bacteriol. *193*, 3162–3163.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D., Alston, M., Stringer, M.F., Betts, R.P., Baranyi, J., et al. (2012). Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. J. Bacteriol. *194*, 686–701.

Sabbagh, S.C., Forest, C.G., Lepage, C., Leclerc, J.M., and Daigle, F. (2010). So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS Microbiol. Lett. *305*, 1–13.

Sharma, C.M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., Chabas, S., Reiche, K., Hackermüller, J., Reinhardt, R., et al. (2010). The primary transcriptome of the major human pathogen *Helicobacter pylori*. Nature *464*, 250–255.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C.M., Rolle, K., Binnewies, T.T., Hinton, J.C., and Vogel, J. (2008). Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet. *4*, e1000163.

Skinner, M.E., Uzilov, A.V., Stein, L.D., Mungall, C.J., and Holmes, I.H. (2009). JBrowse: a next-generation genome browser. Genome Res. *19*, 1630–1638.

Tedin, K., and Bläsi, U. (1996). The RNA chain elongation rate of the lambda late mRNA is unaffected by high levels of ppGpp in the absence of amino acid starvation. J. Biol. Chem. *271*, 17675–17686.

Thattai, M. (2013). Using topology to tame the complex biochemistry of genetic networks. Philos. Trans. A. Math Phys. Eng. Sci. *371*, 20110548.

Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Balestrino, D., Loh, E., Gripenland, J., Tiensuu, T., Vaitkevicius, K., et al. (2009). The *Listeria* transcriptional landscape from saprophytism to virulence. Nature *459*, 950–956.

Tomljenovic-Berube, A.M., Mulder, D.T., Whiteside, M.D., Brinkman, F.S., and Coombes, B.K. (2010). Identification of the regulatory logic controlling *Salmonella* pathoadaptation by the SsrA-SsrB two-component system. PLoS Genet. *6*, e1000875.

Vogel, J., and Luisi, B.F. (2011). Hfq and its constellation of RNA. Nat. Rev. Microbiol. *9*, 578–589.

Wagner, G.P., Kin, K., and Lynch, V.J. (2012). Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory Biosci. *131*, 281–285.

Wagner, G.P., Kin, K., and Lynch, V.J. (2013). A model based criterion for gene expression calls using RNA-seq data. Theory Biosci. *132*, 159–164.

Wang, Q., Frye, J.G., McClelland, M., and Harshey, R.M. (2004). Gene expression patterns during swarming in *Salmonella typhimurium*: genes specific to surface growth and putative new motility and pathogenicity genes. Mol. Microbiol. *52*, 169–187.

Wilhelm, B.T., Marguerat, S., Watt, S., Schubert, F., Wood, V., Goodhead, I., Penkett, C.J., Rogers, J., and Bähler, J. (2008). Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. Nature *453*, 1239–1243.

Wright, J.A., Tötemeyer, S.S., Hautefort, I., Appia-Ayme, C., Alston, M., Danino, V., Paterson, G.K., Mastroeni, P., Ménager, N., Rolfe, M., et al. (2009). Multiple redundant stress resistance mechanisms are induced in *Salmonella enterica* serovar Typhimurium in response to alteration of the intracellular environment via TLR4 signalling. Microbiology *155*, 2919–2929.